Environmental Health Perspectives
Vol. 80, pp. 117-125, 1989

Integrated Control of Proliferation and Differentiation of Mesenchymal Stem Cells

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The physiological control of cellular proliferation and differentiation is an integrated regulatory process. This conclusion is based upon observations using numerous *in vivo* and *in vitro* experimental systems of which murine BALB/c 3T3 T mesenchymal stem cells represent an excellent *in vitro* model. In these cells the coupling of growth arrest and differentiation occurs at a distinct biological state, and this predifferentiation arrest state is distinguishable by a variety of criteria from other restriction points, such as the growth factor deficiency arrest state and the nutrient deficiency arrest state. Most importantly, only cells at this predifferentiation arrest state acquire the potential to differentiate without undergoing DNA synthesis. From this state, differentiation can then occur as a two-step process. Cells first undergo nonterminal differentiation and, second, they terminally differentiate. Nonterminal differentiation is characterized by expression of a completely *differentiated adipocyte phenotype with retention of proliferative potential. Thereafter, when nonterminally* differentiated cells undergo the terminal event in differentiation, they irreversibly lose their proliferative potential. In this paper, data are reviewed which establish that the integrated control of proliferation and differentiation in 3T3 T mesenchymal stem cells is mediated both at the predifferentiation arrest state and at the state of nonterminal differentiation.

Introduction

To establish the mechanisms that regulate the integrated control of proliferation and differentiation, murine BALB/c 3T3 T mesenchymal stem cells have been used as a model *in vitro* system. This stem cell line is valuable for a variety of reasons. In contrast to most other cell differentiation systems, such as the erythroleukemia, neuroblastoma, melanoma, and embryonal carcinoma cells that are transformed (*1–4*), 3T3 T stem cells are nontransformed with respect to the two-stage model of carcinogenesis (*5,6*). They therefore can be used for studies on the mechanisms of the initiation and promotion of carcinogenesis (*6–11*). This is true even though they are immortal and aneuploid. Another valuable characteristic of 3T3 T stem cells is that they possess the ability to differentiate and therein to integrally regulate the control of proliferation and differentiation under defined physiological conditions. Somewhat similar biological characteristics have also recently been reported in normal diploid human keratinocyte progenitor cells (*12–15*).

3T3 T mesenchymal stem cells are thought to be representative of a variety of related cell lines including the 3T3-L1 (*16*), 3T3-F442A (*17*), 3T3-C2 (*18*), ob17 (*19*), and (10T½)TA1 (*20*). When undifferentiated, all such cells have a fibroblastlike morphology, but when they differentiate into specific cell types, such as adipocytes, they modulate not only their morphology but also their function. In this regard, adipocytes were previously considered to be terminally differentiated and therefore incapable of proliferation and cell division. It has, however, now been established that adipocytes as well as some other differentiated tissues can reproduce and undergo differentiation and maturation (*5–11,21,22*). The general phenomena of cell differentiation and the cellular and molecular changes that occur during this process have been elucidated in studies involving these excellent experimental systems and in studies on differentiation into a variety of mesen-

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chymal cell types, including adipocytes, striated muscle cells, chondrocytes, vascular pericytes, and macrophages (*5,23,24*).

In this paper the observations that have been made concerning the differentiation of 3T3 T stem cells into adipocytes will be reviewed, especially the processes involved in the integrated regulation of proliferation and differentiation. The basis for these studies was the development of experimental methods to induce rapid and parasynchronous differentiation of these cells into adipocytes at low density under physiological conditions rather than as in other experimental systems wherein differentiation typically requires prolonged culture of cells at a high density in high concentrations of pathological fluids and/or drugs. More specifically, in 3T3 T stem cells, differentiation into adipocytes can be induced within 8 to 12 days when cultured at $< 1 \times 10^4$ cells/cm^2 in Dulbecco's modified Eagle's medium (DME) containing 25% human plasma and heparin on tissue culture plastic. It has recently been found that culturing 3T3 T stem cells at a similar low density in heparinized DME containing human plasma on nonadherent surfaces (bacteriological Petri dishes) significantly accelerated the kinetics of differentiation and therein more than 95% of the cells differentiate within 5 to 7 days (*25*). Therefore, in 3T3 T stem cells, high cell density and extensive cell-to-cell contact are not required for adipocyte differentiation. Prolonged culture is also not required for adipocyte differentiation nor are high concentrations of serum and/or added hormones, drugs, or chemicals.

With this model cell system it has been possible to definitively establish the biological steps involved in the integrated control of proliferation and differentiation and to show that this control process can be distinguished from other metabolic events that simply block cell growth. Figure 1 presents a summary of the results of these studies in diagrammatic form. It specifically demonstrates that three distinct steps are involved in the integrated control of proliferation and differentiation: predifferentiation growth arrest, nonterminal differentiation, and terminal differentiation. Each of these processes will be discussed in detail in the following sections of this review.

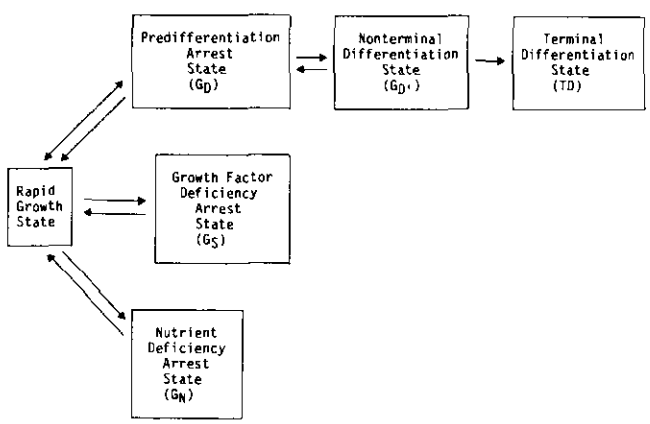

FIGURE 1. Model for the integrated control of 3T3 T mesenchymal stem cell proliferation and differentiation (*50*). The model illustrates the relative organization of the G_D, $G_{D'}$, TD, G_S, and G_N states in the G_1 phase of the cell cycle as discussed in the text. G_D, the predifferentiation growth arrest state; $G_{D'}$, the nonterminal differentiation state; TD, the terminal differentiation state; G_S, the reversible growth arrest state induced by growth factor or serum deprivation; and G_N, the reversible growth arrest state induced by nutrient deprivation.

Predifferentiation Growth Arrest State

To understand the physiological basis for the control of cell proliferation, most previous studies have focused on events that occur in the G_1 phase of the cell cycle (*26–31*). From these studies, it was concluded that there are one or more specific restriction points in G_1 that control cell proliferation. Extensive cell-cell contact or depletion of growth factors or low molecular weight nutrients from the culture medium have been shown to induce transient/reversible growth arrest. A more physiological mechanism to regulate cell proliferation, however, occurs in association with the process of cellular differentiation. This is so because *in vivo* stem cells regulate their proliferation in association with regulation of their differentiation.

The available evidence also suggests that growth arrest in the G_1 phase of the cell cycle is associated with expression of the differentiated phenotype in many cell types (*28–30*). These observations raise the question of whether control of both cell proliferation and differentiation can be mediated at common states in the G_1 phase of the cell cycle. To resolve this initial question, 3T3 T stem cells were growth arrested at various states in G_1 and then tested to determine whether differentiation could be induced from all of growth arrest states or only at a distinct arrest state. To summarize the results, the restriction of cell proliferation by high cell density, serum deprivation, or nutrient deprivation does not support or facilitate the differentiation of 3T3 T stem cells. Rather, these stem cells must growth arrest at a distinct cell cycle state prior to differentiation. (Studies on normal human keratinocyte progenitor cells also demonstrate the requirement for predifferentiation growth arrest, but they do not show as stringent a cell-cycle specificity.)

When low density, rapidly growing 3T3 T stem cells are cultured in DME containing human plasma, they growth arrest in G_1 and remain in this state for approximately 2 to 4 days (*32*). At this predifferentiation growth arrest state, designated G_D, cells have the potential to reinitiate cell proliferation if they are refed a growth factor-enriched medium. Alternatively, if they are refed or maintained in differentiation-promoting medium, up to 95% of the cells convert to adipocytes within 3 to 8 days depending on the cells' microenvironment. In contrast, if cells are growth arrested at high density in serum containing DME or if cells are growth arrested in a growth factor-deficient or nutrient-deficient medium at low cell densities, significant differentiation does not occur even if such cultures are refed differentiation-promoting medium so long as such cells cannot traverse the cell cycle. Therefore, the predifferentiation growth arrest state, G_D, appears to rep-

resent a distinct state in the G_1 phase of the cell cycle in 3T3 T stem cells.

The finding that differentiation occurs without DNA synthesis in 3T3 T stem cells only at the predifferentiation growth arrest state is a significant one because it suggests that at this state specific sets of genes can be preferentially regulated. To substantiate that quantal mitosis and DNA synthesis are not required for adipocyte differentiation, essentially pure populations of undifferentiated metaphase cells were isolated and subsequently placed in differentiation-promoting media under conditions that restricted their capacity to undergo DNA synthesis and cell cycle traverse. At least 80% of 3T3 T stem cells could be induced to differentiate by factors that act solely in late M or G_1 to activate the expression of differentiation-specific genes. That is, under appropriate microenvironmental conditions, metabolic events that occur solely in the late M or G_1 phases of the cell cycle can mediate the integrated control of mesenchymal stem cell proliferation and differentiation at the G_D state.

In this regard, studies were performed to determine if neoplastic transformation is associated with development of a selective defect in one or more of the G_1 growth arrest processes (*33*). Upon examining eight clones and several uncloned tumorigenic 3T3 T stem cell lines, it was initially shown that all the tumorigenic cell lines lacked the ability to undergo predifferentiation growth arrest and to differentiate, whereas some transformed cells did retain the ability to growth arrest at low density when deprived of growth factors and/or nutrients. More detailed recent studies, however, have established that some transformed 3T3 T stem cells can in fact differentiate even though they do express significant defects in the stringency of their differentiation regulatory mechanisms (*8*). In fact, it has been concluded that carcinogenesis is probably associated with the development of defects in the stringency by which proliferation and differentiation are integrally regulated. The characteristics of these defects are also thought to determine whether a cancer is well differentiated and slow growing or poorly differentiated and rapidly growing.

A variety of additional studies have been performed comparing cells arrested at the G_D, G_S, and G_N states (see Fig. 1 for a definition of these terms). These studies all substantiate the conclusion that G_D arrested cells are distinct by a variety of criteria, including the expression of specific biochemical and morphological markers (Fig. 2). For example, when scanning electron microscopy was employed to examine the cell surface characteristics of 3T3 T stem cells at various states in the G_1 phase of the cell cycle, predifferentiation growth arrested cells demonstrated distinct long cell surface microvilli, which were not observed in G_S or G_N arrested cells (*34*).

G_D, G_S, and G_N arrest states were also distinguished in their topography in G_1. Kinetic studies were performed to measure the time required for cells arrested at various G_1 states to initiate synthesis of DNA and to determine the ability of these growth arrested cells to convert from one arrest state to another in the absence of DNA synthesis. Relative to the start of the G_1 phase of the cell cycle, a topographical sequence was established in the following order: G_D, G_S, G_N (*35*).

Cells at the predifferentiation growth arrest state are also characterized by their unique sensitivity to the mitogenic effect of isobutyl methyl xanthine (MIX). When the relative ability of a variety of mitogens to induce DNA synthesis of cells in the G_D and G_S arrest states was compared, cells at the G_D arrest state, regardless of cell density, could be induced to proliferate by MIX treatment, whereas cells at the G_S arrest state at both high and low density could not be stimulated (*36*). Other characteristics of cells at the predifferentiation growth arrest state not exhibited by G_S- and G_N-arrested cells include their predilection to form extensive cell-cell clusters, a high sensitivity to the cytotoxic effect of 8-bromocyclic AMP (*37*), and the expression of more than 100 distinct polypeptides detected in two-dimensional gel electrophoretic analysis.

One of the most important questions that must now be resolved concerns the identification of the physiological factors that mediate predifferentiation growth arrest and the underlying mechanisms that are involved. Although such factors are yet to be purified, preliminary studies indicate that a specific protein factor(s) present in human plasma and serum acts to induce growth arrest at the predifferentiation arrest state, and this factor has tentatively been designated the G_D arrestor. It is acid and heat labile, trypsin sensitive, and dithiothreitol (DTT) resistant. This protein(s) does not absorb to an Affi-gel blue matrix, but does bind to heparin agarose. Its isoelectric point is ≤ 7.0, with a molecular weight $> 14{,}000$. Interestingly, the effects of this protein can be blocked by tumor promoters such as TPA. Addition of TPA (≤ 100 ng/mL) to medium containing human plasma inhibits the induction of predifferentiation growth arrest.

In summary, these results suggest that the coupling of growth arrest and differentiation can occur at a distinct state in 3T3 T stem cells. Once cells arrest their growth at this predifferentiation state, they acquire the potential to differentiate if they are not first induced to reinitiate proliferation by the addition of mitogenic factors. Thus, predifferentiation growth arrest is a pivotal state because at this state cells either differentiate or reinitiate proliferation depending on the availability of growth factors or differentiation factors in the microenvironment.

Nonterminal Differentiation State

Once predifferentiation growth arrest has occurred, what additional steps are involved in the process of differentiation? Does a single early event in the differentiation process program cells to lose their proliferative potential in association with differentiation, or does a stable nonterminal state of differentiation exist? Many previous observations suggest that a state of nonterminal differentiation does indeed exist in many cell types, but an experimental model system to study this process has been lacking. *In vivo* studies on cardiac myocytes and neuroblasts show that during development many cells that are terminally differentiated in the adult animal actually pass through a nonterminal phase during development

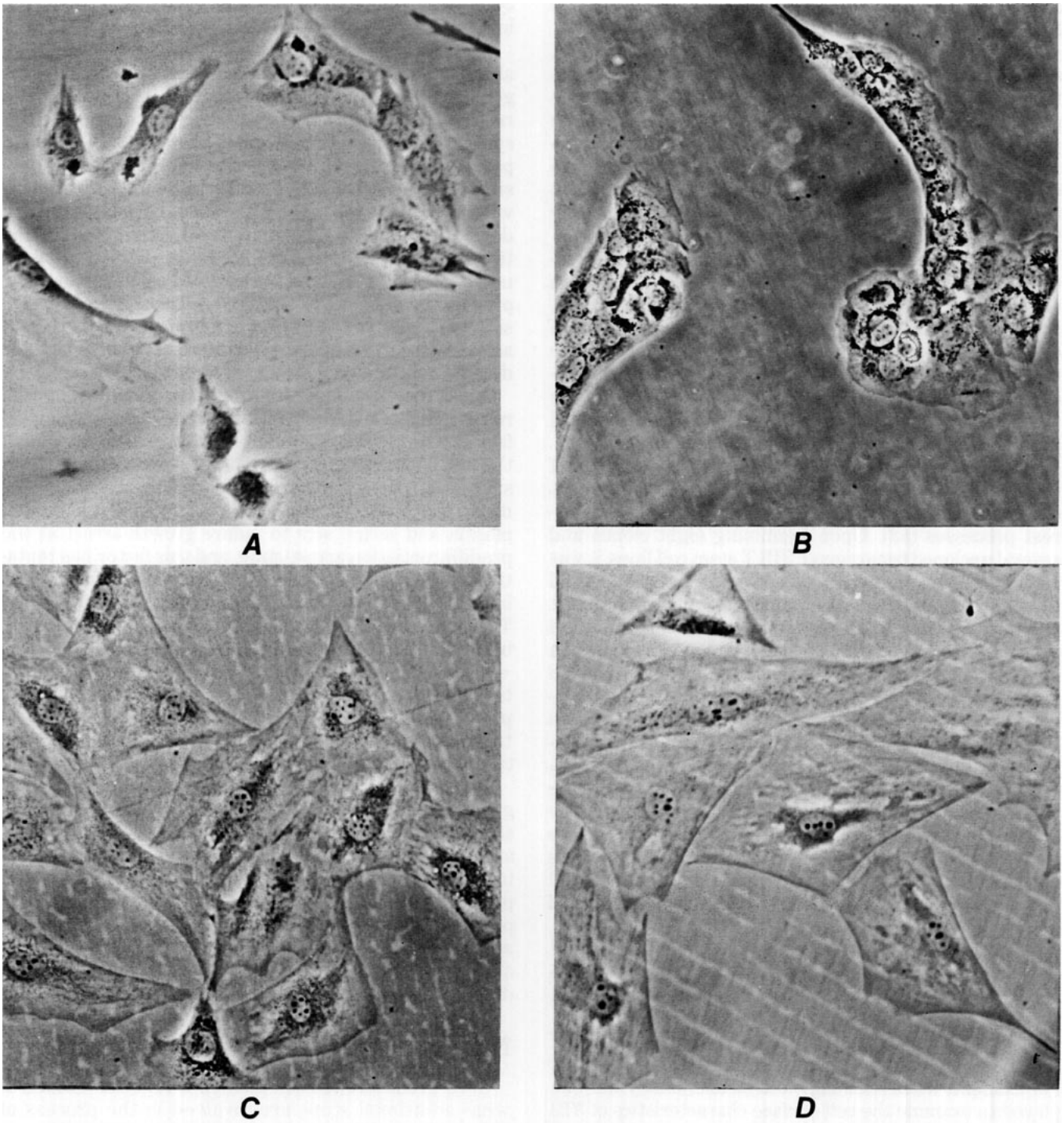

FIGURE 2. Phase-contrast micrographs of 3T3 T mesenchymal stem cells at different growth vs. growth arrest states. Rapidly growing cells in 10% FCS (*A*) cells at the predifferentiation growth arrest state (G_D) after 4-day culture in 25% human plasma (*B*); cells at the growth factor deficiency arrest state (G_S) after 4-day culture in 0.5% FCS (*C*); cells at the nutrient deficiency growth arrest state (G_N) after 3-day culture in isoleucine-deficient DME containing 10% dialyzed FCS (*D*).

(*38,39*). Lymphocytes, liver cells, Schwann cells, and smooth muscle cells are also all maintained in a nonterminally differentiated state for extended periods even in adult tissues before terminal differentiation occurs (*40–43*). *In vitro* studies also suggest that a nonterminal differentiation state exists in some cell types. For example, myoblasts that have withdrawn from the cell cycle and express myosin can be induced to reenter the cell cycle when exposed to growth media (*44*). Similar observations have been reported in various neoplastic cells including mast cells (*45*), melanoma cells (*46*), erythroleukemia cells exposed to hemin (*1*), and neuroblastoma cells exposed to

butyrate (*47*). Nonetheless, the process of nonterminal differentiation has not been studied in detail nor has the terminal event in differentiation. 3T3 T stem cells were therefore evaluated in this regard.

Experimental conditions were developed that limited the terminal phase of differentiation in 3T3 T adipocytes and thereby enrich cultures in nonterminally differentiated cells. The culture of low density 3T3 T stem cells in heparinized DME containing a plasma fraction, designated citrate eluate of the barium-precipitated plasma (CEPH), achieved this result (*48,49*). Such cultures first underwent predifferentiation growth arrest, and flow microfluorometric analysis showed these cells to have the following cell cycle distribution: G_1, 87%; S, 3%; G_2/M, 10%. Thereafter, these cultures differentiated into adipocytes that retained their proliferative potential for extended intervals and therefore exist in a nonterminally differentiated state. Proof of this was documented by the demonstration that 80 to 90% of such adipocytes were capable of undergoing DNA synthesis and clonal proliferation (Fig. 3). In the 3T3 T mesenchymal stem cell system, expression of the differentiated phenotype and loss of proliferative potential are therefore separately controlled processes; the expression of the differentiated phenotype is not sufficient to induce loss of proliferative potential.

The development of experimental conditions to induce and purify adipocytes at the nonterminal states of differentiation is a significant one (*48*). While the previous *in vivo* and *in vitro* studies mentioned above provided evidence for the existence of a nonterminally differentiated state and the transition of such cells to a subsequent terminal state of differentiation, with the 3T3 T stem cell system it has been possible to actually perform well-controlled studies on the transition from nonterminal to terminal states of differentiation. Thus, this model provides an excellent system to examine the separate and specific control processes associated with differentiation *per se* and those processes associated with loss of proliferative potential. These will be discussed in the next section of this review.

Numerous biochemical changes are associated with the induction of adipocyte differentiation. These include an increase in the glucose uptake and a significant increase in the concentration of enzymes involved in lipogenesis. These enzymes include lipoprotein lipase, fatty acid synthetase, diacylglycerolacyltransferase, phosphatidic acid phosphase, glycerol-3-phosphate dehydrogenase (G3PD), and acetyl COA carboxylase (*16–21*). In the 3T3 T cell system, the specific expression of the nonterminal adipocyte phenotype has also been shown to be associated with the accumulation of intracellular lipid droplets and increased activity of G3PD and lipoprotein lipase (*49*). In fact, there is a very good correlation in the kinetics of nonterminal differentiation using assays for the activity of lipoprotein lipase, glycerol-3-phosphate dehydrogenase, and presumably all other lipogenic enzymes and morphological assays.

The process of nonterminal differentiation can be induced by a variety of factors including proteins in blood and various hormones. In addition, it can be modulated by TGF-β (*50*). That is, while other biologically active polypeptides, such as EGF, NGF, and somatomedin C, have no specific effect on 3T3 T adipocyte differentiation even at high concentrations, TGF-β is a potent reversible inhibitor of adipocyte differentiation (50% inhibition at 0.06–0.08 ng/mL). TGF-β actually inhibits differentiation in a cell cycle-dependent manner by its effect on a specific phase of the differentiation process. That is, TGF-β blocks the transition from the predifferentiation growth arrest state to the nonterminal differentiation state.

During the process of nonterminal differentiation, cells not only acquire a completely differentiated phenotype but they also downregulate their response to growth factors. This phenomena does not prevent the nonterminally differentiated cells from proliferating; it simply requires that they be exposed to a high concentration of appropriate mitogens. For example, whereas predifferentiation growth arrested cells can be induced to proliferate with 5% fetal calf serum (FCS), nonterminally differentiated adipocytes require 30% FCS plus 50 μg/mL insulin to express a maximum mitogenic response. The integrated control of cell proliferation and differentiation is therefore mediated not only at the predifferentiation growth arrest state but also at the nonterminal differentiation state.

This finding answers a question that previously has confounded investigators studying the control of cell differentiation. Do cells have to undergo a process designated commitment prior to terminal differentiation? With respect to the 3T3 T model stem cell system, it appears that there is no specific commitment step occurring that makes cells differentiate terminally because neither reversible arrest at the predifferentiation state nor nonterminal differentiation predisposes cells subsequently to undergo the terminal event in differentiation and thereby lose their proliferative potential (*24*). Previous theories proposing that loss of proliferative potential results from an inhibitory effect of differentiation gene products on DNA synthesis, on cellular proliferation, and/or on cell division therefore cannot hold true in these cells because a stable, nonterminally differentiated state clearly exists and it can be experimentally maintained and modulated.

To reiterate, the characteristics of adipocytes at the nonterminal state of differentiation include: a) the potential to make decisions to integrate the control of differentiation and proliferation; b) the expression of a fully differentiated phenotype; c) the retention of proliferative potential; and d) the expression of decreased responsiveness to growth factors. In addition, nonterminally differentiated adipocytes can be induced to dedifferentiate and subsequently redifferentiate into another cell type (*23,24*). That is, studies have specifically demonstrated that nonterminally differentiated adipocytes can be induced to lose the fat cell phenotype without undergoing DNA synthesis when treated with retinoic acid, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or methyl isobutyl xanthine (*48*). Such cells that have lost the adipocyte phenotype yet remain in the G_1 phase of the cell cycle and retain most of the characteristics of cells in predifferentiation growth arrest thereafter become responsive to environmental influences that can further induce their redifferentiation into adipocytes without DNA synthesis or their prolifer-

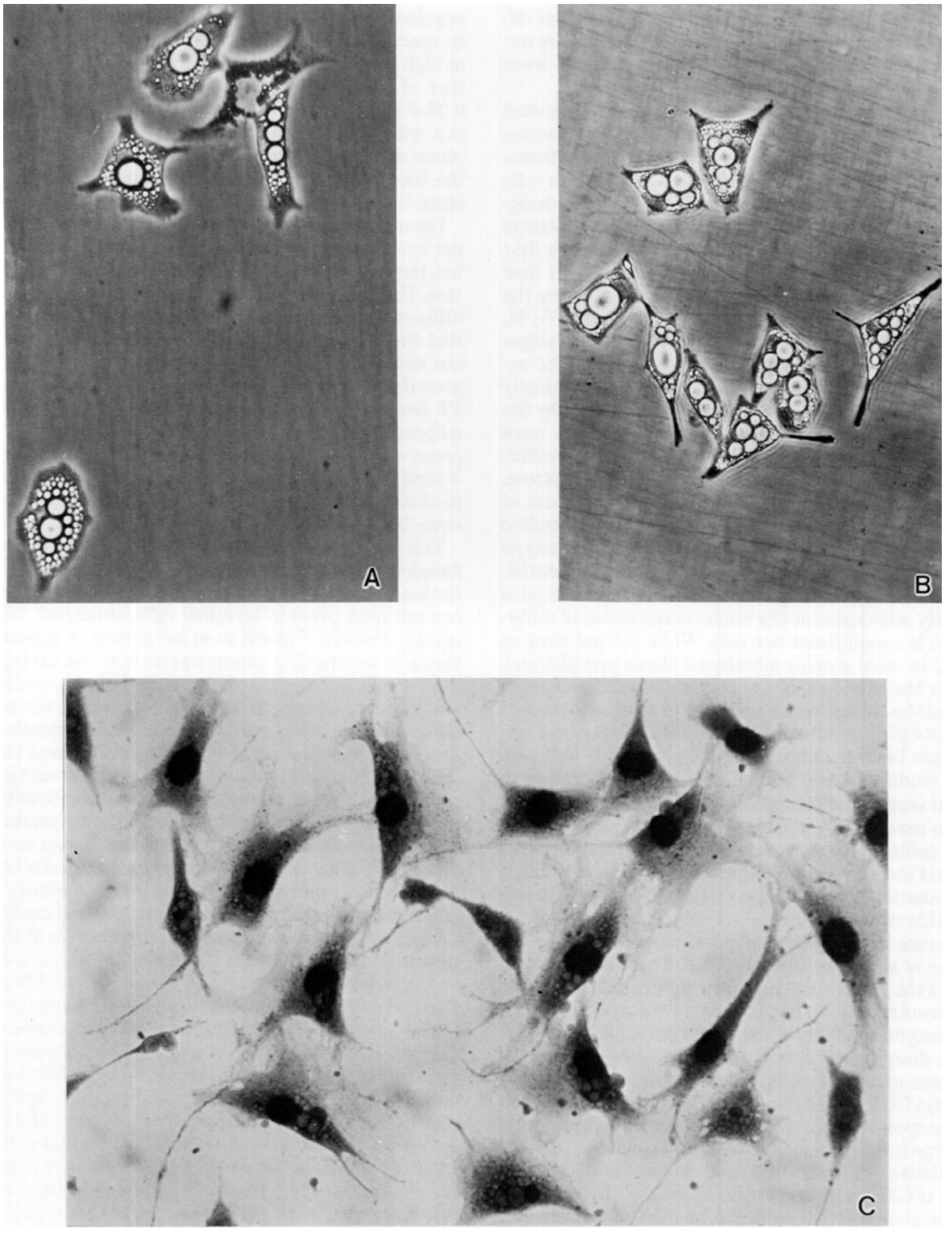

FIGURE 3. Phase-contrast micrographs of 3T3 T mesenchymal stem cells at different differentiation states: adipocytes at the nonterminal differentiation state (G_{D1}) (*A*) and adipocytes at the terminal differentiation state (TD) (*B*). An autoradiograph of adipocytes at the nonterminal differentiation state following incubation in ^{3}H-thymidine to measure DNA synthesis is also illustrated (*C*). The figure shows that nonterminally differentiated adipocytes can undergo DNA synthesis.

ation and/or differentiation into another cell type (*24*).

In summary, the studies reviewed demonstrate that following growth arrest at the predifferentiation state, two steps are involved in adipocyte differentiation. First, the growth arrested cells undergo nonterminal differentiation wherein they develop an adipocyte morphology yet retain their proliferative potential. Thereafter, terminal differentiation occurs wherein differentiated cells specifically lose their proliferative potential. Most important, these results establish that the integrated control of proliferation and differentiation can be mediated at the nonterminal state of differentiation.

Terminal Differentiation State

The control of cellular proliferation is an important regulatory process. In normal stem cells, it appears to be mediated by a balance of forces that either stimulate proliferation or stimulate nonterminal or terminal differentiation. Most previous experimental studies on the control of cellular proliferation have examined the mechanisms by which DNA synthesis and cellular proliferation are initiated in quiescent cell populations. In related studies, many growth factors and several growth inhibitory factors have been characterized and purified. Concerning the latter, these factors generally inhibit DNA synthesis and/or cell proliferation in a transient, reversible manner.

Few studies have attempted to establish the mechanisms by which a cell's proliferative potential can be irreversibly limited in nonterminally differentiated cell populations. The reason is because few, if any, adequate model cell systems have previously been developed wherein nonterminally differentiated cells can be rapidly and parasynchronously induced to terminally differentiate and irreversibly lose their proliferative potential when exposed to a physiological inducer. In this respect, the 3T3 T stem cell system provides an excellent model with which to study the terminal event in cellular differentiation. In this cell system, distinct nonterminal and terminal states of differentiation can be identified, and transition from the nonterminal to the terminal states of differentiation can be induced by a component of human plasma (*48,49*).

What are the characteristics of the terminal event in cellular differentiation and how do nonterminal and terminal cells differ? A series of biological, biochemical, and molecular studies have been performed to define as carefully as possible the answer to these questions and to establish the mechanisms that mediate the irreversible loss of the proliferative potential.

Nonterminally and terminally differentiated populations of 3T3 T adipocytes exhibit an essentially identical morphology, they express similar levels of adipogenic enzymes, and they are similarly growth arrested in the G_1 phase of the cell cycle (Fig. 3). A reasonable assumption therefore is that they differ only in their proliferative capacity. Since such cells are very similar, studies were performed to determine if any protein differences between these cells could be observed and whether these differences might be associated with loss of proliferative potential associated with the terminal event in differentiation. By analysis of silver-stained two-dimensional gel electrophoretograms, a very limited number of differences in the composition of major proteins between nonterminally and terminally differentiated adipocytes were indeed detected. Out of more than 1000 protein spots evaluated, only 6 were selectively expressed in terminally differentiated cells; of these, 2 were localized to the cytoplasm, and 4 were localized to the nucleus (*51*). Another distinct protein was selectively identified in the nucleus of nonterminally differentiated adipocytes.

More recently, three distinct basic nuclear proteins have been identified in nonterminally differentiated cells that are selectively lost during the process of terminal differentiation. These proteins were identified by the use of cross-reactive monoclonal antibodies to heat shock protein 90 (unpublished observations). Additional studies are currently in progress to further characterize the structure and function of these proteins as are experiments to clone and characterize their genes. In this regard, it is important to note that these observations indicate that the terminal event in the process of mesenchymal stem cell differentiation is probably associated with modulation in the expression of less than 1% of the major cytosolic and nuclear proteins.

The significance of these differences in the expression or abundance of specific proteins in nonterminally and terminally differentiated adipocytes is still an open question. The differences could be due to primary changes in gene expression at the two states of differentiation or to changes in the rate of protein synthesis/degradation. Variations in posttranslational protein modification such as glycosylation and/or acetylation might also account for some of the observed differences. Posttranslational protein modification via differential phosphorylation is, however, not likely to be involved in this process since significant differences were not observed in recent studies evaluating the phosphoproteins in nonterminally and terminally differentiated adipocytes (unpublished observations). The most significant question that must now be resolved concerns which specific proteins function to control cellular proliferation and how they can irreversibly cause cells to lose proliferative potential.

It is also important to establish which proteins in human plasma actually induce transition from the nonterminal to terminal state of differentiation. This goal has been partially achieved by the 20,000-fold purification of a component of human plasma that induces the terminal event in adipocyte differentiation (*52*). This factor has an apparent molecular weight of 45,000 daltons and an isoelectric point of approximately 7.6. It is trypsin sensitive, acid and heat labile, and is resistant to treatment with dithiothreitol and alkali. The ability of this human plasma protein to induce the irreversible loss of proliferative potential associated with the terminal event in adipocyte differentiation serves as the basis for its designation "aproliferin" (*52*). In this regard, none of the more than 20 pharmacologic or physiologic agents that have been examined can mimic the biologic effect of aproliferin. Therefore, it appears that aproliferin is a functionally distinct

protein that can convert nonterminal adipocytes to terminal adipocytes within 12 hr of exposure. The mechanism by which aproliferin mediates this effect is, however, still under investigation. Experiments are also in progress to prepare anti-aproliferin monoclonal antibodies, to sequence aproliferin, and to clone the gene for this potentially important protein.

Additional studies have furthermore established that the terminal event in differentiation also can be reversibly inhibited by tumor necrosis factor (TNF). That is, whereas TGF-β selectively inhibits the transition from predifferentiation growth arrest to nonterminal differentiation, TNF selectively inhibits the transition from nonterminal differentiation to terminal differentiation (unpublished observations). Evidence has also been obtained that suggests that aberrant 3T3 T stem cell clones that cannot terminally differentiate actually secrete molecules that function in a manner similar to TNF to block their own terminal differentiation in an autocrine and/or paracrine manner.

To summarize, adipocytes that undergo the terminal event in differentiation are characterized by two most important features. They cannot reinitiate proliferation nor can they be induced to lose their adipocyte phenotype. During terminal differentiation, adipocytes appear to acquire six distinct polypeptides not present in nonterminally differentiated cells and to lose at least four proteins that are present in nonterminally differentiated adipocytes. It also appears that these changes are induced by exposure of cells to aproliferin. By definition, terminally differentiated cells therefore do not mediate any aspect of the integrated control of proliferation and differentiation because they cannot modify their differentiation nor proliferation even though they are viable cells.

Resume and Speculation

Figure 1 again summarizes the results of our studies that establish the biological processes by which 3T3 T mesenchymal stem cells integrally regulate control of their proliferation and differentiation. The data show that in these stem cells multiple cell cycle restriction points exist but that the integrated control of proliferation and differentiation is mediated only at a distinct predifferentiation growth arrest state and at the state of nonterminal differentiation. The results also define the characteristics of these states and the fact that nonterminally differentiated cells can be specifically induced to undergo the terminal event in differentiation following exposure to the human plasma protein designated aproliferin.

A series of additional questions now need to be resolved specifically concerning whether all multipotential stem cells demonstrate regulatory mechanisms comparable to 3T3 T mesenchymal stem cells and/or whether progenitor cells show a different regulatory mechanism. Although our model for the integrated control of proliferation and differentiation in 3T3 T stem cells has been confirmed in studies on neural cells (*53*), myogenic cells (*54*), and embryonal carcinoma cells (*4*), we speculate that some cell types may have evolved somewhat distinct regulatory mechanisms. We also speculate that progenitor cells that have a more restricted differentiation and/or proliferation potential may in fact be found to integrally regulate proliferation and differentiation in a distinct manner.

The challenge for the future is to clearly define the options cells have to control their proliferation and differentiation and to determine what specific defects in these regulatory mechanisms occur during carcinogenesis and other disease processes.

This research was supported by the Mayo Foundation, by NIH grants CA 28240, CA 21722, and CA 09441, and by grants from the Eagles. The authors thank the previous members of our laboratory who have contributed to these studies, including Drs. Dagne Florine, Bruce Boman, Bruce Krawisz, Marjorie Wier, Rodney Sparks, Kankatsu Yun, and John Wille. The authors also thank Karen Connelly for excellent assistance in the typing of this manuscript.

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